THE EFFECT OF SOME REAGENTS ON THE "FILTRATE FACTOR" (A WATER-SOLUBLE VITAMIN BELONGING TO THE VITAMIN B COMPLEX AND PREVENTING A DIETARY DERMATITIS IN CHICKS)

BY SAMUEL LEPKOVSKY AND THOMAS H. JUKES

(From the Division of Poultry Husbandry, University of California, Berkeley and Davis)

(Received for publication, January 27, 1936)

Kline, Keenan, Elvehjem, and Hart (1) have described a syndrome produced in chicks by feeding a heated diet of natural foodstuffs. Elvehjem and Koehn (2) have described the preparation from liver of a concentrate which will prevent the syndrome. The concentrate was prepared from an aqueous extract of liver from which the flavins had been removed by adsorption on fullers' earth and filtration. It is proposed for the sake of brevity to refer in this communication to the factor present in the concentrate as the "filtrate factor," in reference to the method of preparation by its discoverers (1, 2). The name intentionally leaves in abeyance the question of the identity or non-identity of the factor with the rat factor vitamin B_6 (3). It has been suggested by Stare (4) that the two factors may be identical, but György (3) reported vitamin B_6 to be destroyed by visible light, while Elvehjem and Koehn (2) reported that the chick factor was not destroyed by exposure to light.

EXPERIMENTAL

Method of Assay

The care of the chicks, the production of the syndrome, and the method of scoring for intensity of symptoms have been previously described (5). It was found desirable to introduce the following modifications.

1. Vitamin G (lactoflavin) was added to the diet in the form of a fullers' earth adsorbate of whey corresponding to 60 per cent of

whey. In a few experiments 5 per cent of skim milk powder which had been heated at 120° for 24 hours to destroy the filtrate factor was used instead. This was found necessary because it was shown previously (5, 6) that the basal unheated diet of Elvehjem and coworkers may be deficient in vitamin G (flavin). It is obviously necessary to insure that an experimental diet is as far as possible complete in all essentials except the factor under study.

2. The antihemorrhagic vitamin, which has been demonstrated to be necessary for the chick (7), was added to the diet in the form of a hexane extract of alfalfa meal. The extract was evaporated on the diet. The level fed corresponded to 1 per cent of alfalfa meal. The addition abruptly checked a tendency to hemorrhagic symptoms which had been occasionally observed. The hemorrhagic symptoms may have been aggravated by the fact that the birds were rigorously excluded from access to their droppings, since Almquist and Stokstad have observed (unpublished data) that the antihemorrhagic vitamin is present in chick droppings.

3. The period on the stock diet (5) was 10 days, and the depletion period on the diet heated at 120° was from 5 to 12 days. Chicks appeared to be less vigorous in the summer and fall months, and a shorter depletion period was used during these times.

The birds were weighed individually and symptoms noted at intervals during the assay period of 2 weeks. The average gain per bird for a period of 2 weeks on the supplemented diet was compared with the gain by the control group on the basal diet. Ten birds were used in each group. The "syndrome score" (5) was roughly correlated with weight changes but was found to be too variable for use in assaying the filtrate factor.

Experiments with Crude Liver Filtrate

Extract of beef liver (5) was shaken with fullers' earth to remove vitamins B and G, and filtered. The liver filtrate was used for the following experiments.

Effect of Autoclaving Followed by Treatment with Fullers' Earth—A sample of liver filtrate was autoclaved at its natural pH of about 5 at 20 pounds pressure for 30 minutes, and shaken with fullers' earth and filtered. The fullers' earth was inactive, and the potency of the filtrate was undiminished.

Extraction with Butyl Alcohol—A sample of liver filtrate was

acidified with hydrochloric acid and submitted to continuous extraction with butyl alcohol in the apparatus described by Dakin (8). The factor was extracted, but the process was slow, and was accompanied by darkening.

Attempt with Adsorption with Lead Sulfide—A sample of liver filtrate was treated with lead chloride and hydrogen sulfide. The precipitate of lead sulfide was removed by filtration. The potency of the filtrate was undiminished, and the filtrate factor could not be detected in a sodium hydroxide eluate of the lead sulfide precipitate.

Experiments with Concentrated Extract Prepared from Liver Filtrate

It has been shown by Elvehjem and Koehn (2) that the filtrate factor distributes itself between amyl alcohol and water in a proportion governed by the pH of the aqueous phase. This observation was made use of in devising a method for preparing concentrated solutions of the factor. Liver filtrate was brought to a pH of between 1 and 2 and shaken with repeated changes of isoamyl alcohol. The combined amyl alcohol layers were shaken with three changes of dilute sodium hydroxide to extract the factor. The resultant aqueous solution was brought to a pH of about 4 and concentrated under reduced pressure to a convenient volume. The solution was added to the heated diet and assayed biologically. Aliquots of the solution were then submitted to chemical treatments, and fed at a level which was usually twice as great as the level of the original solution found necessary to produce the maximum growth response.

Precipitation of Impurities with Barium Hydroxide—Excess of saturated barium hydroxide solution was added and the mixture allowed to stand overnight at 0°. The precipitate was separated, and the precipitate and the filtrate were fed separately after removal of barium with sulfuric acid. The precipitate did not contain the factor, which passed into the filtrate without perceptible loss. The same result was obtained when barium hydroxide precipitation was made in 80 per cent alcoholic solution, which led to a removal of an even greater amount of inert material.

Fractionation with Lead Acetate—60 cc. of concentrate were diluted to 200 cc. and 60 cc. of saturated lead acetate solution were added. The mixture was cooled with ice and ammonium hydrox-

ide was slowly added, with stirring, until a pH of 6.6 was reached. The precipitate was removed, dissolved in acetic acid, and reprecipitated at pH 6.6. The combined filtrates were brought to pH 8.0, and the precipitate removed, dissolved in acetic acid, and reprecipitated at pH 8.0. The filtrates were combined, and all fractions were freed from lead by addition of a little hydrochloric acid and excess of hydrogen sulfide. The factor was absent from the precipitates at pH 6.6 and pH 8.0, and present in the filtrate from precipitation at pH 8.0, although about half of the potency had disappeared.

Attempts at Inactivation with Oxidizing Agents. Nitrous Acid—30 cc. of concentrate were acidified with HCl and placed in a boiling water bath. 15 cc. of 5 per cent sodium nitrite were added over a period of 20 minutes, followed by 15 cc. of 7.5 per cent urea solution. A slight diminution in potency was brought about by the treatment. Another aliquot of the concentrate was diluted with 1 volume of concentrated HCl, and cooled to 0°. 1 volume of 35 per cent sodium nitrite solution was added, the temperature being kept below 5°. The mixture was allowed to stand overnight at 0°, and excess of urea was then added. The solution was aerated at room temperature and boiled at 40° under reduced pressure. A slight diminution in potency was brought about by the treatment.

Bromine—2.5 volumes of saturated bromine water were added to the concentrate. The mixture was allowed to stand for 1 hour at room temperature and then aerated to remove free bromine. The precipitate was discarded, and the filtrate was fed. No diminution in potency was detected. The experiment was repeated, but the bromine was removed by aeration at 60°. A slight diminution in potency was found.

Nitric Acid—4 cc. of nitric acid were added to 30 cc. of the concentrate. The mixture was heated to 60° and allowed to cool spontaneously. It was neutralized with sodium hydroxide and fed. No diminution in potency was detected.

Attempt at Adsorption with Ferric Hydroxide—Three aliquots of 20 cc. of the concentrate, Samples 1, 2, and 3, were used. To Samples 1 and 2, 0.75 gm. of ferric chloride were added, and to Sample 2 was added a slight excess (14 cc.) of 1 N sodium hydroxide solution. An equal amount of sodium hydroxide solution was

added to Sample 3. All three aliquots were heated in a boiling water bath for a few minutes to flocculate the precipitate in Sample 2. The precipitate and filtrate in Sample 2 were separated, the precipitate redissolved in dilute hydrochloric acid, and the filtrate acidified. Sample 3 was acidified, and all solutions were fed. Ferric chloride alone caused no inactivation. Sodium hydroxide alone caused a partial inactivation. No potency was detected in the filtrate from ferric hydroxide precipitation, or in the redissolved ferric hydroxide precipitate.

Attempt at Adsorption with Charcoal—An aliquot of the concentrate was treated with acid-washed norit according to Kinnersley *et al.* (9), with 12 gm. of charcoal per 100 cc. The filtrate from charcoal was colorless and potent. The yellowish acid-alcohol eluate ("Peters' eluate") of charcoal was not potent.

Behavior of Charcoal Filtrate towards Oxidizing and Reducing Agents—Separate aliquots of the charcoal filtrate of the preceding paragraph were treated with cold bromine water, with 0.2 volume of 30 per cent hydrogen peroxide solution, and with sodium bisulfite according to Williams *et al.* (10). In no case could reduction of potency be detected.

Removal of Inert Material from Aqueous Extract of Rice Bran

Concentrated aqueous rice bran extract (5), freed from vitamins B and G by treatment with fullers' earth, was diluted with 10 volumes of methanol and the resultant precipitate discarded. The filtrate was concentrated *in vacuo* to small bulk. A portion of this aqueous solution was mixed with 2 volumes of methanol and 6 volumes of acetone to precipitate inert material. The factor remained in solution. Another portion was mixed with 2 volumes of methanol and 3 volumes of secondary amyl alcohol. The factor remained in solution.

Demonstration of Deficiency of Filtrate Factor in Unheated Diet of Purified Foodstuffs

8 day-old chicks were fed a diet of starch, washed casein, vitamin B adsorbate prepared by shaking rice bran extract with fullers' earth, vitamin G adsorbate prepared similarly from whey, paper pulp, salt mixture (4), and cod liver oil. Growth was slow, and typical dermatitis appeared in about 10 days. Liver filtrate was

then added to the diet, and growth improved almost immediately. Dermatitis began to disappear, but within 3 weeks nearly all of the chicks were dead, owing to lack of the antiencephalomalacic vitamin (11, 12).

DISCUSSION

The filtrate factor differs markedly from vitamins B and G in its failure to be adsorbed readily from acid solution by fullers' earth or charcoal. In contrast to vitamin B, it is not destroyed by autoclaving; while in contrast to vitamin G, it is destroyed by dry heat at 120° for 24 hours, which does not destroy vitamin B (13). The factor is noteworthy for its resistance to oxidizing and reducing agents and its solubility in moist, weakly polar solvents.

Dermatitis was produced on an unheated diet of purified foodstuffs and cured by the filtrate factor. The diet was incomplete, but the experiment served to emphasize that the dermatitis caused by the heated diet is due to a deficiency rather than to production of injurious substances in the diet of natural foodstuffs by the heat treatment.

Block and Hubbell (14) have recently presented evidence that the third factor of the vitamin B complex, essential for the rat, is adsorbed on Lloyd's reagent and is eluted by dilute sodium hydroxide. Experiments completed in this laboratory confirm this result, and indicate that an extract of rice bran may be separated into two fractions by means of treatment with fullers' earth; the unadsorbed fraction is more potent for the chick, when fed with the heated diet, and the adsorbed fraction is more potent for the rat.

We prefer to retain the nomenclature vitamin G for flavin, in common with Bisbey and Sherman (15) and with Block and Hubbell (14). Experiments with crystalline lactoflavin and hepatoflavin (6) have indicated that the growth-promoting effect of whey adsorbate and liver adsorbate when added to chick diets adequately supplemented with the filtrate factor is due to vitamin G (flavin).

SUMMARY

1. The name "filtrate factor" is provisionally applied to the water-soluble vitamin which has been demonstrated by workers at

the University of Wisconsin to prevent the dermatitis produced in chicks by feeding a heated diet of natural foodstuffs (1).

2. The factor was not precipitated by barium hydroxide in aqueous or 80 per cent alcoholic solution, and was not destroyed by bromine water, ferric chloride, dilute nitric acid, sodium bisulfite, hydrogen peroxide, or hydrogen sulfide. Nitrous acid brought about a slight diminution in potency. The factor was partially inactivated by warming with sodium hydroxide, and completely inactivated by warming with a mixture of ferric chloride and sodium hydroxide.

3. Norit, lead sulfide, and fullers' earth failed to adsorb the factor appreciably from acid solution.

4. Fractional precipitation with lead acetate failed to yield the factor in the precipitates at pH 6.6 and pH 8.0, and a large part of the original potency was found in the filtrate from the precipitation at pH 8.0.

5. Concentrated solutions of the factor were prepared from an aqueous extract of rice bran by treatment with fullers' earth, methanol, and acetone or secondary amyl alcohol.

6. Dermatitis in chicks was also produced by feeding a purified diet, and cured by addition of the filtrate factor to the diet.

7. Indications have been found that, by means of treatment of an aqueous extract of rice bran with fullers' earth, the third factor of the vitamin B complex, essential for the rat, may be at least partially separated from the filtrate factor, which supplements the heated diet for the chick.

Our thanks are due to Vitab Products, Inc., of San Francisco, for supplying us with whey adsorbate (used as a source of vitamin G) and rice bran extract; to Armour and Company, Chicago, for liver extract; to the California Milk Products Company, Gustine, for casein; and to the F. E. Booth Company for the loan of battery brooders. The assistance of K. K. Miya and S. Grushkowitz is gratefully acknowledged. Facilities of the Animal Husbandry Division were kindly placed at our disposal by Dr. George H. Hart.

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J. Biol. Chem. 1936, 114:109-116.